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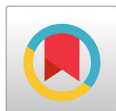
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RESEARCH ARTICLE

# Development and Validation of a Bioanalytical Method for the Simultaneous Determination of 14 Antiretroviral Drugs using Liquid Chromatography-Tandem Mass Spectrometry



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**OBJECTIVES:** The aim was to develop and validate a LC-MS/MS assay to determine antiretrovirals in human plasma for routine therapeutic drug monitoring.

**METHODS:** The selectivity, sensitivity, linearity, accuracy, precision, recovery, matrix effect, stability and dilution integrity and carry-over were validated according to EMA and FDA standards.

**RESULTS:** For accuracy and precision, the highest overall bias was 11.3% at LLOQ of both lopinavir and saquinavir. The highest overall CV was 15.6% at the LLOQ of darunavir. Storage stability at 4°C, 20–25°C and 10°C had a maximum CV of 13.2% at low QC level (0.2 mg/L) for saquinavir. Freeze-thaw stability had a maximum overall bias of 7.4% at low QC level (0.8 mg/L) for tipranavir. Selectivity and specificity showed no interfering peaks of more than 20% of the LLOQ.

**CONCLUSIONS:** The bioanalytical method is suitable for both TDM in standard care and clinical studies.

**KEYWORDS:** lc-ms/ms, antiretrovirals, hiv, TDM.

## INTRODUCTION

The treatment of Human Immunodeficiency Virus (HIV) has improved significantly over the last three decades. This improvement was entirely due to the broad introduction of antiretroviral (ARV) drugs in a combined treatment regimen (cART) [1]. With the advent of cART the mortality and morbidity associated with HIV-1 infection

and acquired immunodeficiency syndrome (AIDS) sharply decreased [2]. ARV drugs are designed to intervene in the HIV replicative cycle, which eventually results in an HIV ribonucleic acid (RNA) load decrease and subsequently, the recovery of the host immune system. Currently, 25 ARV drugs have been approved for the treatment of HIV by the Food and Drug Administration (FDA) and the European Medicines Agency (EMA) [3]. These drugs are divided in six classes, each class representing a target in the HIV life cycle: Nucleoside Reverse Transcriptase Inhibitors (NRTI), Nonnucleoside Reverse Transcriptase Inhibitors (NNRTI), Protease Inhibitors (PI), Fusion Inhibitors (FI), Entry Inhibitors and integrase strand transfer inhibitors (INI) [3].

In order to optimize treatment outcome and to prevent drug resistance, therapeutic drug monitoring (TDM) is recommended in cases of: drug-drug interaction; renal or hepatic morbidity; pregnancy; administration of drug doses not commonly used; virologic failure; suspicion of nonadherence and adverse events [4]. In order to be eligible for TDM a correlation should exist between drug concentrations and effect or adverse effects; or, a drug should have large inter-individual pharmacokinetic variability; or a narrow therapeutic index [5]. In daily practice at this point NNRTIs, PIs and INIs qualify for TDM [5-7]. Previously, several assays have been published describing the simultaneous determination of ARV drugs for TDM [8-11]. The analytical methods described often used UV detection or included an extensive sample preparation such as liquid-liquid extraction. These methods are time-consuming and incur high costs. Liquid chromatography (LC) tandem mass spectrometry (MS) is considered to be the most important analytical technique which is superior to UV detection due to its specificity [12]. Further, using a stable isotope internal standard (IS) for LC tandem MS is highly recommended as it corrects for injection and ionization variability [13]. A stable isotope IS will compensate for these deviations and ensures a robust, high-throughput assay [13].

The aim of this study was to develop and validate a bioanalytical assay to determine both older and new PIs and NNRTIs, and two INIs with a simple sample preparation and using stable isotope IS followed by LC-MS/MS analysis in human plasma as this approach is more cost-effective, less time-consuming than most methods using UV detection or extensive sample preparation.

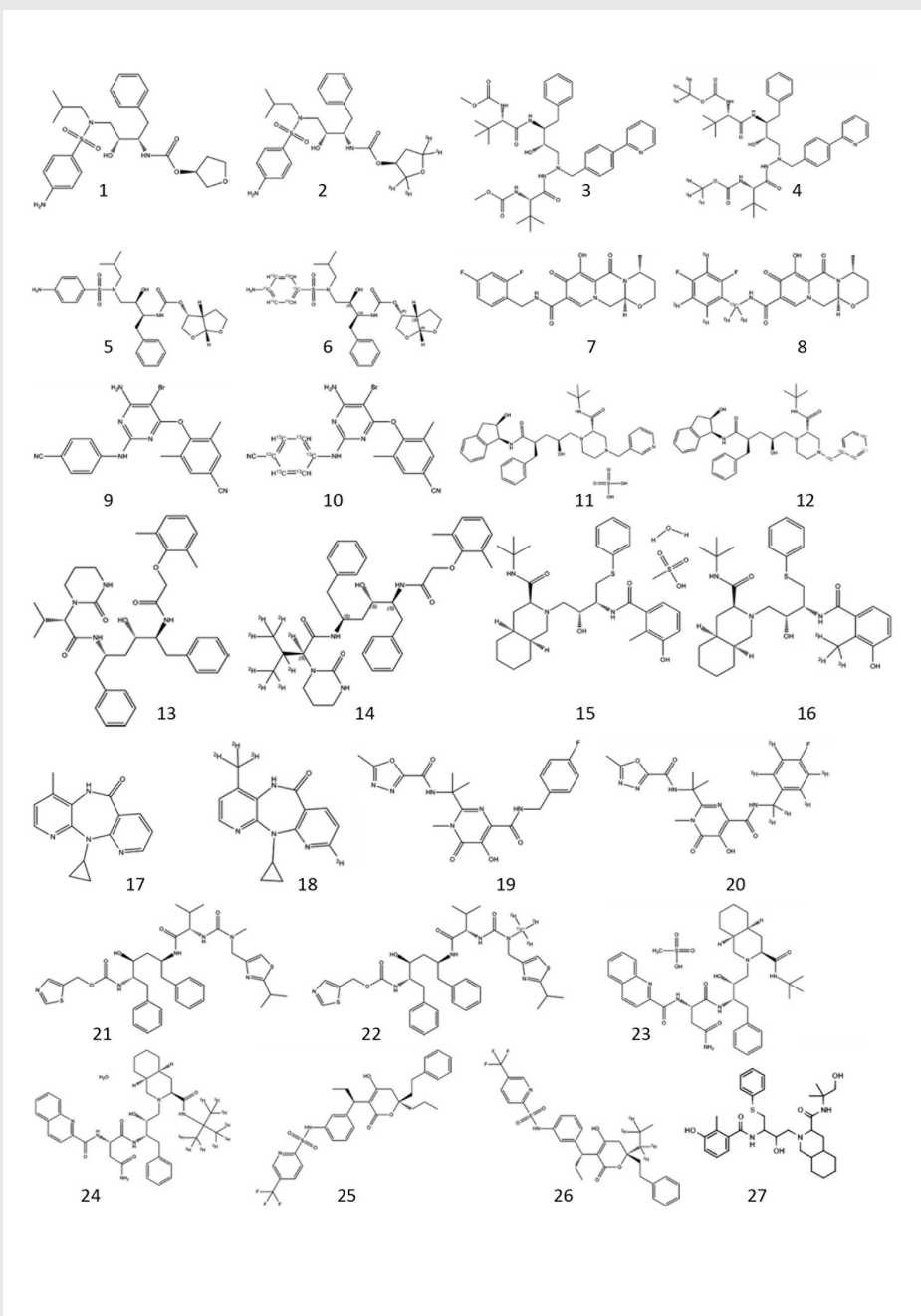
## MATERIALS AND METHODS

### Analysis

The following ARVs were included in the method: amprenavir, atazanavir, darunavir, dolutegravir, etravirine, indinavir, lopinavir, nelfinavir, nelfinavir-M8, nevirapine, raltegravir, ritonavir, saquinavir and tipranavir. The corresponding stable isotope IS were used: [ $^2\text{H}_4$ ]-amprenavir, [ $^2\text{H}_6$ ]-atazanavir, [ $^{13}\text{C}_6$ ]-darunavir, [ $^{13}\text{C}, ^2\text{H}_5$ ]-dolutegravir, [ $^{13}\text{C}_6$ ]-etravirine, [ $^{13}\text{C}_4, ^{15}\text{N}$ ]-indinavir, [ $^2\text{H}_8$ ]-lopinavir, [ $^2\text{H}_3$ ]-nelfinavir, [ $^2\text{H}_4$ ]-nevirapine, [ $^2\text{H}_6$ ]-raltegravir, [ $^{13}\text{C}, ^2\text{H}_3$ ]-ritonavir, [ $^2\text{H}_9$ ]-saquinavir and [ $^2\text{H}_4$ ]-tipranavir. The ARVs and IS used (**Figure 1**), were purchased from Alsachim (Illkirch, Graffenstaden, France), except for nelfinavir-M8 which was purchased from Pfizer Inc. (New York, United States of America). Acetonitrile for LC-MS/MS was purchased from BioSolve (Valkenswaard, The Netherlands). The chemicals used, including methanol and trifluoroacetic acid, were of high-pressure liquid chromatography (HPLC) or analytical grade and were purchased from VWR (Amsterdam, The Netherlands). Purified water was obtained from a Milli-Q water purifying system (Millipore Corporation, Billerica, MA, USA).

Both pooled human serum samples and pooled human plasma samples with ethylene diamine tetraacetic acid (EDTA) as anticoagulant (Becton Dickinson Vacutainer®,  $\text{K}_2\text{E}$  7.2 mg) were made available in accordance with the standard operating procedures of our medical center.

One milligram of each stable isotope IS was added to methanol to a concentration of 1 g/L and stored at  $-80^\circ\text{C}$ . The precipitation reagent consisted of a mixture of methanol Li-chrosolv, acetonitrile (4:21, v/v) and IS. Twenty-five microliters per IS was standard spiked



**Figure 1.** Chemical structures of the analytes and stable isotope internal standards used in the current bioanalytical method.

1 = Amprenavir, 2 = [ $^2\text{H}_4$ ]-amprenavir, 3 = Atazanavir, 4 = [ $^2\text{H}_6$ ]-atazanavir, 5 = Darunavir, 6 = [ $^{13}\text{C}_6$ ]-darunavir, 7 = Dolutegravir, 8 = [ $^{13}\text{C}$ ,  $^2\text{H}_3$ ]-dolutegravir, 9 = Etravirine, 10 = [ $^{13}\text{C}_6$ ]-etravirine, 11 = Indinavir (sulphate), 12 = [ $^{13}\text{C}_4$ ,  $^{15}\text{N}$ ]-indinavir, 13 = Lopinavir, 14 = [ $^2\text{H}_8$ ]-lopinavir, 15 = Nelfinavir (mesylate monohydrate), 16 = [ $^2\text{H}_3$ ]-250nelfinavir, 17 = Nevirapine, 18 = [ $^2\text{H}_4$ ]-nevirapine, 19 = Raltegravir, 20 = [ $^2\text{H}_6$ ]-raltegravir, 21 = Ritonavir, 22 = [ $^{13}\text{C}$ ,  $^2\text{H}_3$ ]-ritonavir, 23 = Saquinavir (mesylate), 24 = [ $^2\text{H}_9$ ]-251saquinavir, 25 = Tipranavir, 26 = [ $^2\text{H}_4$ ]-tipranavir, 27 = Nelfinavir-M8

**Table 1.** Gradient elution.

Time (min)	A (%)	B (%)	C (%)
0.00	5	90	5
0.40	5	90	5
0.41	5	45	50
1.80	5	37.5	57.5
1.81	5	0	95
2.50	5	0	95
2.51	5	90	5
2.90	5	90	5

A: aqueous buffer, B: purified water, C: acetonitrile.

directly in 250 mL precipitation reagent. To 10 µL of each sample, a volume of 750 µL of precipitation reagents combined with the IS was added to a vial. The samples were vortexed for 1 min. The vials were centrifuged for 5 min at 9500g. Ten microliters of the upper layer was injected into the LC-MS/MS.

The analysis was performed on a triple-quadrupole LC-MS/MS (Thermo Scientific, San Jose, CA, USA) with an MS Pump Plus (Finnigan Surveyor) and autosampler Plus (Finnigan Surveyor). The mass spectrometer was a triple-stage quadrupole Quantum Access Max mass spectrometer. The autosampler temperature was set at 10°C. Liquid chromatographic separation was performed on a HyPURITY C18 analytical column (50 by 2.1 mm, 3 µm particle size; Thermo Scientific, Interscience, Breda, The Netherlands), and the temperature was set at 20°C. The mobile phase had a flow rate of 0.5 mL/min and consisted of purified water, acetonitrile, and an aqueous buffer (containing ammonium acetate [5 g/L], acetic acid [35 mL/L of water], and trifluoroacetic acid [2 mL/L of water]). The method had a run time of 2.9 min and chromatographic separation was performed by means of an elution gradient shown in **Table 1**. Peak area ratios of the ARVs and their IS were used to calculate concentrations.

The MS was operating in positive electrospray ionization mode and selected reaction monitoring (SRM) mode with a positive spray voltage of 5,000V, a capillary temperature of 350°C, a vaporizer temperature of 150°C and a sheath gas pressure and auxiliary pressure of 60 and 10 arbitrary units, respectively. High-purity argon was used for both the source and collision gas flows and high-purity nitrogen for the sheath gas. The mass transitions used in ARV drug quantification and the detail mass spectrometry conditions are demonstrated in **Table 2**. A scan width of 0.5 m/z was used for all components. Peak area integration for all components was calculated by Xcalibur software version 2.0.7 (Thermo Fisher, San Jose, CA, USA).

### Method validation

The analytical method was validated in accordance with the guidance for Industry of the FDA and the EMA guidelines [14,5]. For the validation of the assay the selectivity, sensitivity, linearity, accuracy, precision, recovery, matrix effect, stability and dilution integrity were determined.

For all components separate stock solutions were made in a concentration of 10 g/L, with the exception of tipranavir; for this drug a stock solution of 20 g/L was made. All stock solutions were diluted to 500 mg/L, except for the stock solution of tipranavir that was diluted to 2 g/L. Dimethylsulfoxide (DMSO) was used as solvent for dilution. Subsequently 1, 2, 5, 10, 25, 50, 100, 150 and 200 µL of each diluted stock solution was spiked to 5 mL EDTA plasma, resulting in a final composition of 4% organic solvent in the calibration

**Table 2.** Mass transitions and detail mass spectrometry conditions.

Component	Drug class	Parent ion (m/z)	Product ion (m/z)	SRM collision energy (eV)	Typical retentiontime (min)
Nevirapine	NNRTI	267.1	226.1	25	1.30
[ <sup>2</sup> H <sub>4</sub> ]-Nevirapine		271.1	230.1	26	1.30
Indinavir	PI	614.3	364.1	29	1.45
[ <sup>13</sup> C <sub>4</sub> , <sup>15</sup> N]-Indinavir		619.3	470.2	23	1.45
Dolutegravir	INI	420.1	277.0	26	1.50
[ <sup>13</sup> C, <sup>2</sup> H <sub>5</sub> ]-Dolutegravir		426.1	277.0	27	1.50
Nelfinavir-M8	PI	584.4	330.0	33	1.50
Nelfinavir		568.3	467.2	26	1.85
[ <sup>2</sup> H <sub>3</sub> ]-Nelfinavir		571.3	470.2	28	1.85
Raltegravir	INI	445.1	360.9	17	1.55
[ <sup>2</sup> H <sub>6</sub> ]-Raltegravir		451.2	367.1	17	1.55
Saquinavir	PI	671.3	433.2	42	1.60
[ <sup>2</sup> H <sub>9</sub> ]-Saquinavir		680.4	433.2	42	1.60
Amprenavir	PI	506.2	418.1	12	1.75
[ <sup>2</sup> H <sub>4</sub> ]-Amprenavir		510.2	418.2	13	1.75
Darunavir	PI	548.2	392.1	14	1.75
[ <sup>13</sup> C <sub>6</sub> ]-Darunavir		554.3	398.1	13	1.75
Atazanavir	PI	705.4	335.1	28	2.00
[ <sup>2</sup> H <sub>6</sub> ]-Atazanavir		711.4	338.1	28	2.00
Ritonavir	PI	721.2	197.0	39	2.20
[ <sup>13</sup> C, <sup>3</sup> H <sub>5</sub> ]-Ritonavir		725.3	201.0	38	2.20
Lopinavir	PI	629.3	429.2	22	2.30
[ <sup>2</sup> H <sub>8</sub> ]-Lopinavir		637.4	429.2	22	2.30
Etravirine	NNRTI	435.0	303.9	37	2.50
[ <sup>13</sup> C <sub>6</sub> ]-Etravirine		441.1	310.0	37	2.50
Tipranavir	PI	603.2	333.0	29	2.60
[ <sup>4</sup> H <sub>4</sub> ]-Tipranavir		607.2	334.1	29	2.60

NNRTI: Non-Nucleoside Reverse Transcriptase Inhibitor, PI: Protease Inhibitor, INI: Integrase Inhibitor

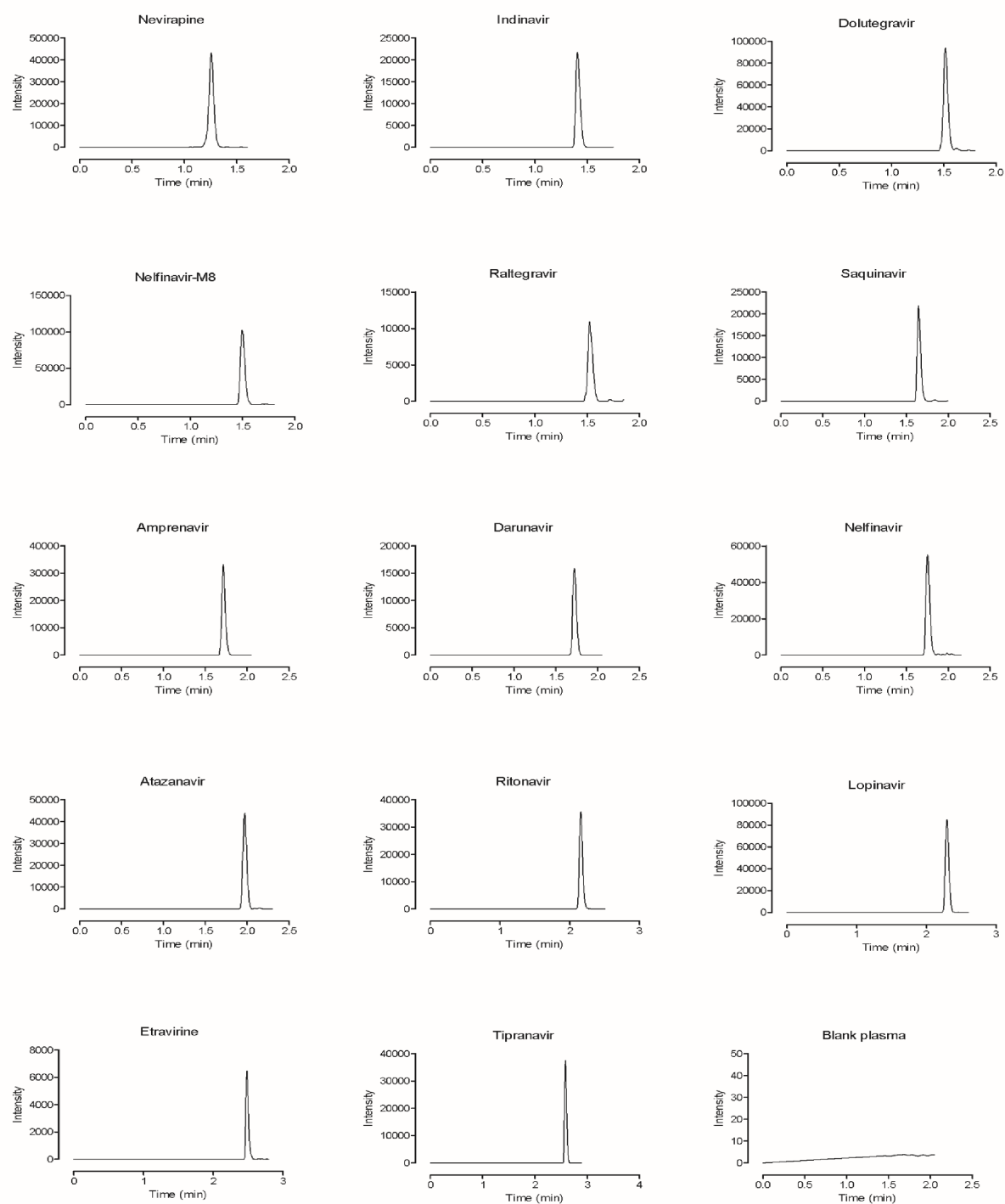
standards. The analysis for linearity was conducted in 5 replicates per concentration. The selection of the assay range was based on the utilization for TDM in daily practice. The calibration standards, blanks, and quality control (QC) samples were fully thawed at room temperature. The criteria used for the selection of the QC concentration levels were based on FDA and EMA guidelines [14,15], LOW was 2 or 3 times the LLOQ; MED was at 40% and HIGH was at 80%. QC samples and calibration standards were stored at -20°C. QC samples with 4 different concentrations of each ARV were used. The concentrations and number of samples used for the calibration curves in combination with the concentrations used for the QC samples per component are shown in **Table 3**.

**Table 3.** Concentrations of calibration standards and QC samples.

Component	Number of samples	Calibration curves	LLOQ	QC samples mg/L		
		Concentrations (mg/L)		LOW	MED	HIGH
Amprenavir Darunavir Etravirine Lopinavir	8	0.2, 0.5, 1.0, 2.5, 5.0, 10.0, 15.0, 20.0	0.2	0.5	8.0	16.0
Atazanavir Indinavir Nelfinavir Nelfinavir-M8 Nevirapine Ritonavir Saquinavir	9	0.1, 0.2, 0.5, 1.0, 2.5, 5.0, 10.0, 15.0, 20.0	0.1	0.2	8.0	16.0
Dolutegravir Raltegravir	6	0.2, 0.5, 1.0, 2.5, 5.0, 10.0	0.2	0.5	4.0	8.0
Tipranavir	9	0.4, 0.8, 2.0, 4.0, 10.0, 20.0, 40.0, 60.0, 80.0	0.4	0.8	32.0	64.0

QC: quality control, LLOQ: lower limit of quantitation

For selectivity, 6 pooled human plasma samples were examined for interference and their responses were compared with those of the LLOQ samples. Over 3 days, each day a single calibration curve in plasma was analysed and accuracy was measured by evaluation of five determinations per QC sample on three consecutive days. Precision was divided into within-run and between-run values using the same method as used for the accuracy. The coefficient of variation (CV) for the LLOQ was maximized at 20% deviation and the CV for the other QC samples should not exceed 15%. The recovery was determined on three levels (LOW, MED, and HIGH) and was done in five replicates. The stabilities of the ARVs were tested for storage stability and freeze-thaw stability. Storage stability of the ARVs was examined by storing QC samples at room temperature (20°C to 25°C) in a refrigerator at 4°C and after sample preparation in the autosampler at 10°C, all for 7 days. Stability was also tested using five freeze-thaw cycles at -20°C. All stability tests were done using two different QC levels (LOW and HIGH) in five determinations per concentration. The FDA does not describe a maximum CV requirement for stability, therefore a maximum CV of 15% was used for the current method in accordance with the EMA guidelines [14]. In order to determine potential differences between the analysis of ARVs in human plasma and in human serum, a matrix comparison was performed. Since protein precipitation was the single required sample preparation step in this method, relative recovery was measured by comparing the ratios of integrated peak area of the ARVs and the corresponding IS of the QC samples processed with the average peak area of the recovery samples. Recovery samples (low, med, and high) were post-extraction blank samples spiked at the same concentrations as the QC samples. To determine the dilution integrity, on three consecutive days, for each ARV drug a sample was diluted 10 times and then prepared in five replicates. To determine the carry-over a blank sample



**Figure 2.** Chromatograms of the included antiretrovirals and the corresponding stable isotope internal standards at the LLOQ and blank plasma.



**Table 4.** Concentrations of calibration standards and QC samples

Component	Correlation coefficient (linear range) mg/L	Slope	Intercept	Concentration (mg/L)	Within Run CV(%)	Between Run (CV(%))	Overall CV(%)	Overall Bias (%)
Amprenavir	0.999 (0.200–20.0)	0.091	-0.002	LLOQ (0.2)	12.0	8.9	15.0	-4.7
				Low (0.5)	6.7	0.0	6.7	-4.5
				Medium (8.0)	3.7	3.0	4.8	0.0
Atazanavir	0.999 (0.100–20.0)	0.115	-0.00141	High (16.0)	2.7	3.0	4.1	-0.9
				LLOQ (0.1)	7.7	0.0	7.7	-8.0
				Low (0.2)	4.3	1.2	4.5	-8.3
Darunavir	0.998 (0.200–20.0)	0.146	-0.002	Medium (8.0)	2.6	3.5	4.4	1.3
				High (16.0)	1.1	3.4	3.6	1.8
				LLOQ (0.2)	16	0.0	16	-2.0
Dolutegravir	0.999 (0.200–10.0)	0.167	0.0	Low (0.5)	6.8	2.6	7.3	-3.9
				Medium (8.0)	3.9	5.1	6.4	0.3
				High (16.0)	3.4	6.1	7.0	-0.1
Entravirine	0.998 (0.200–20.0)	0.141	0.0	LLOQ (0.2)	5.4	0.0	5.4	-5.0
				Low (0.5)	4.8	1.1	4.9	-9.1
				Medium (4.0)	1.8	1.2	2.2	-3.6
Indinavir	0.998 (0.100–20.0)	0.091	0.0	High (8.0)	1.9	0.0	1.9	-4.4
				LLOQ (0.2)	12	7.5	14	-0.7
				Low (0.5)	9.4	0.0	9.4	-1.7
Lopinavir	0.999 0.200–20.0	0.140	0.002	Medium (8.0)	4.5	6.4	7.8	1.2
				High (16.0)	3.2	3.1	4.5	1.3
				LLOQ (0.1)	10	0.0	10	-9.3
Nelfinavir	0.999 (0.100–20.0)	0.099	0.0	Low (0.2)	6.6	0.0	6.6	-9.0
				Medium (8.0)	2.2	2.0	3.0	1.1
				High (16.0)	1.9	3.0	3.5	-0.9
				LLOQ (0.2)	4.7	4.8	6.7	11
				Low (0.5)	4.0	1.8	4.4	1.7
				Medium (8.0)	1.8	3.8	4.2	1.0
				High (16.0)	1.3	3.7	3.9	0.2
				LLOQ (0.1)	9.3	0.0	9.3	-4.0
				Low (0.2)	6.1	0.0	6.1	-2.7
				Medium (8.0)	2.5	2.4	3.4	1.9
				High (16.0)	1.8	3.4	3.9	0.9

QC: quality control, LLOQ: lower limit of quantitation, CV: coefficient of variation, LLOQ: low limit of quantification, testing for linearity, accuracy and precision were conducted in 5 replicates.

**Table 4.** Concentrations of calibration standards and QC samples (continued)

Component	Correlation coefficient (linear range) mg/L	Slope	Intercept	Concentration (mg/L)	Within Run CV(%)	Between Run (CV(%))	Overall CV(%)	Overall Bias (%)
Nelfinavir_M8	0.999 (0.100-20.0)	0.158	0.0	LLOQ (0.1)	8.7	0.0	8.7	-4.0
				Low (0.2)	4.2	3.6	5.5	-0.3
				Medium (8.0)	3.4	2.1	4.0	-0.4
				High (16.0)	1.9	2.8	3.4	-1.0
Nevirapine	0.999 (0.100-20.0)	0.335	-0.002	LLOQ (0.1)	5.0	5.0	7.1	-4.0
				Low (0.2)	4.7	2.1	5.2	-3.3
				Medium (8.0)	1.6	3.0	3.4	2.1
				High (16.0)	0.8	2.2	2.4	1.4
Raltegravir	0.998 (0.200-10.0)	0.143	-0.001	LLOQ (0.2)	12.0	0.0	12	-2.3
				Low (0.5)	9.6	0.0	9.6	-2.3
				Medium (4.0)	5.8	0.0	5.8	2.2
				High (8.0)	7.1	0.0	7.1	3.6
Ritonavir	0.999 (0.100-20.0)	0.158	-0.001	LLOQ (0.1)	4.7	2.9	5.5	-4.0
				Low (0.2)	4.1	3.6	5.4	-5.3
				Medium (8.0)	1.2	3.9	4.1	-0.7
				High (16.0)	1.4	4.5	4.7	-0.5
Saquinavir	0.997 (0.100-20.0)	0.103	0.0	LLOQ (0.1)	9.4	1.6	9.6	11
				Low (0.2)	7.1	0.0	7.1	1.0
				Medium (8.0)	2.1	2.2	3.0	-1.3
				High (16.0)	2.8	2.6	3.8	-1.1
Tipranavir	0.999 (0.400-80.0)	0.183	-0.005	LLOQ (0.4)	5.3	0.0	5.3	-0.3
				Low (0.8)	5.2	1.9	5.6	2.3
				Medium (32.0)	2.0	3.5	4.1	4.5
				High (64.0)	3.0	3.1	4.3	2.3

QC: quality control, LLOQ: lower limit of quantitation, CV: coefficient of variation, LLOQ: low limit of quantification, testing for linearity, accuracy and precision were conducted in 5 replicates.

**Table 5.** Stability testing results at LOW en HIGH concentrations for the included components

Component	Concentration (mg/L)	Refrigorator 4°C (bias) %	Room temp. (bias) %	As stab. 10 °C (bias)%	F/T 5 (bias) %
Amprenavir	Low (0.5)	2.4	6.4	-8.8	-2.9
	High (16.0)	1.3	0.5	-0.9	-3.0
Atazanavir	Low (0.2)	0.0	11	-4.0	0.0
	High (16.0)	1.3	4.0	-0.2	-2.0
Duranavir	Low (0.5)	-6.0	0.4	7.0	-5.4
	High (16.0)	0.4	2.7	4.8	0.0
Dolutegravir	Low (0.5)	-4.8	0.8	-6.4	-6.3
	High (8.0)	0.6	-0.7	-6.1	-2.0
Etravirine	Low (0.5)	-7.6	10	0.5	-1.8
	High (16.0)	0.9	2.6	0.5	-4.0
Indinavir	Low (0.2)	-8.0	6.0	-12	0.6
	High (16.0)	1.1	2.3	-1.0	-1.0
Lopinavir	Low (0.5)	2.8	10	0.4	0.7
	High (16.0)	-0.9	1.9	0.1	-3.0
Nelfinavir	Low (0.2)	2.0	10	0.0	6.6
	High (16.0)	-0.8	1.1	0.8	0.0
Nelfinavir_M8	Low (0.2)	-2.0	1.0	-6.0	0.4
	High (16.0)	-6.8	1.1	-2.9	-3.0
Nevirapine	Low (0.5)	10	12	-2.0	1.2
	High (16.0)	4.2	6.1	2.5	3.0
Raltegravir	Low (0.5)	3.2	-1.6	-3.2	-3.7
	High (8.0)	7.1	4.7	4.8	1.0
Ritonavir	Low (0.2)	-4.0	6.0	-5.0	1.2
	High (16.0)	0.2	0.8	-0.2	-2.0
Saquinavir	Low (0.2)	10	13	2.0	6.0
	High (16.0)	-0.4	-2.3	0.1	-5.0
Tipranavir	Low (0.8)	4.0	12	-2.0	7.4
	High (64.0)	6.9	4.7	1.0	2.0

Temp: temperature, AS stab: autosampler stability, F/T 5: stability of 5freeze and thaw cycles

was analysed after analysing the highest calibration standard per component.

### Clinical application

After the method was validated and approved it was used for TDM in standard care. For use in daily practice blood was collected in a EDTA blood collection tube. Whole blood was centrifuged with 9500 x g for 5 min and a minimum of 0.500 mL plasma was transferred to a vial and stored until analysis in a – 80°C refrigerator. For analysis 10 µL of plasma was transferred to a vial and 750 µL of precipitation reagent was added. The sample was vortexed for 1 min and subsequently centrifuged for 5 min at 9500 x g. Ten microliters of the upper layer was injected into the LC-MS/MS. One calibration curve was used containing all 14 components. The performance of the assay is routinely evaluated by participation in the international quality control program of ARV drugs of the Association for Quality Assessment in Therapeutic Drug Monitoring and Clinical Toxicology

**Table 6.** Components analysed in the period from January 2016 until May 2017 for therapeutic drug monitoring (total = 403 samples).

Component	Number of samples	Median (IQR) concentration (mg/L)
Atazanavir	50	1.4 (0.9-1.9)
Darunavir	142	3.0 (2.1-4.8)
Etravirine	7	0.6 (0.5-0.8)
Lopinavir	65	7.7 (3.9-10.9)
Nevirapine	62	5.6 (3.9-7.2)
Ritonavir	1	3.1 <sup>#</sup>
Raltegravir	75	0.75 (0.2-1.8)
Saquinavir	1	0.5 <sup>#</sup>

<sup>#</sup>: 1 sample

(KKGT) [7,16].

### Statistics

Results were analysed using one-way analysis of variance (ANOVA) in validated Excel sheets (Microsoft, Redmond, WA).

## RESULTS

### Method development

For this bioanalytical method several points of consideration were identified regarding mobile- and stationary phase, sample pre-treatment, MS parameters and usability in daily clinical practice. Earlier studies showed adequate liquid-liquid extraction [8,10,17], however simple sample precipitation was preferred for the current method since it is less time-consuming and less expensive [18]. The precipitation reagent has been used for many other bioanalytical methods in the current composition, among others for the determination of azole antifungals and ertapenem [19,20], and therefore was found to be suitable for this bioanalytical method. In order to obtain adequate retention of the highly polar ARVs an analytical column with high stability under aqueous mobile phase conditions was required. The HyPURITY C18 analytical column has a good polar retention capacity and showed adequate peak shape, retention time and has been extensively used in our daily routine making it a sufficient column for bioanalytical determinations in daily practice in combination with the mobile phase used in a stepwise gradient as demonstrated in **Table 1**. Since the particle diameter of the HyPURITY C18 analytical column was 3 µm the flow rate of the mobile phase was set at 0.5 mL/min in order to ensure optimized resolution and retention time (**Figure 2**). When the flow rate of the mobile phase was set at 0.5 mL/min it was important to set the detector capillary temperature at 350°C. The adjustments of these parameters was necessary to maximize the ionization and subsequently the sensitivity of the detector.

### Method validation results

The validation results for linearity, accuracy and precision are demonstrated in **Table 4**. For accuracy and precision, the highest overall bias found during the validation was 11.3% at LLOQ of both lopinavir and saquinavir. The highest overall CV was 15.6% at the LLOQ of darunavir. The results for the storage- and freeze-thaw stability are displayed in **Table 5**. Storage stability in a refrigerator (4°C), at room temperature (20 – 25°C) and in

the autosampler (10°C) met the requirements with a maximum CV of 13.2% at low QC level (0.2 mg/L) for saquinavir. Furthermore, freeze-thaw stability was within FDA and EMA margins, with a maximum overall bias of 7.4% at low QC level (0.8 mg/L) for tipranavir. Selectivity and specificity showed no interfering peaks of more than 20% of the LLOQ. For the matrix comparison the integrated peak height ratio of a component and its corresponding IS in plasma was compared to that in serum and no statistically significant difference between the two matrices was found for each component. Recovery samples (LOW, MED, HIGH) were assayed and recovery of all components was higher than 85%, with the most deviating recovery of 93.4% at low QC level for etravirine. The dilution integrity was proven with a maximum overall bias of 5% for raltegravir. The carry-over testing showed that there was no carry-over. All results of the method validation were within the EMA and FDA guidelines.

### Clinical application results

This method has successfully been used in clinical practice for TDM in cases of drug-drug interactions, renal or hepatic morbidities, pregnancy, virologic failure, suspicion of nonadherence and adverse events and it was also used in a clinical study on darunavir pharmacokinetics [21]. In the period from January 2016 until May 2017 403 plasma samples were analysed using this bioanalytical method (Table 6). Performance of the assay was within the accepted margins (accuracy and precision <20%) of the international quality control program of ARV drugs of the KKGt in our laboratory.

### DISCUSSION AND CONCLUSION

The bioanalytical method described here is rapid and simple and provides an efficient tool for TDM of ARV drugs. This bioanalytical method contains both new ARVs as older ARVs such as tipranavir, saquinavir, indinavir and nelfinavir, making it also suitable for resource limited settings where these drugs are currently commonly used. We were struck by the good bias and precision data illustrating the robustness of our analysis.

Previously, other comparable assays have been published describing the simultaneous determination of PIs, NNRTs and INIs [8, 10, 17, 22]. Three of these studies required a time consuming liquid-liquid extraction and did not include stable isotope IS [8, 10, 17], what could result in a poorer compensation for inefficiencies in extraction and sample preparation steps, as well as for any matrix effect. One of these bioanalytical methods contained 17 ARVs, but required liquid-liquid extraction and did not include stable isotope IS [8]. All four of these studies had a relatively long run time varying from 10 – 30 min. For TDM purposes a relatively small sample volume and simple sample preparation is desirable. Therefore, the method described by Kromdijk et al. appears to be the best applicable in daily practice compared to the other three methods published [22]. The current described bioanalytical method has a shorter run time and includes more ARVs which can be determined simultaneously than the study of Kromdijk et al. Due to the simple sample preparation, which only included protein precipitation using a precipitation reagent, no time-consuming and expensive liquid-liquid extractions and solid-phase extractions were required. The short run time of 2.9 min, the use of small volumes of plasma and the possibility of the simultaneous determination of 14 ARV drugs makes the method we described highly suitable for routine service. The currently developed method and the applicability for routine service is supported by a recent study by Baldelli et al. [18] where a bioanalytical method for elvitegravir was developed similar to the current one. Baldelli et al. utilized the quality by design approach for the method development which entails a systematic method for quality standards by looking at the entire development system and product life cycle [18].

In the current bioanalytical method overlapping retention times of some components were demonstrated. For the development of this method we achieved a high resolution on the one hand and a fast run-time on the other hand. However, due to its desired applicability

for routine service to clinicians, and the high sensitivity and discriminatory power of LC-MS/MS, a faster run-time prevailed over higher resolution.

The bioanalytical method was simple, specific, robust, reproducible and demonstrated a high sensitivity for all components and better cost-effectiveness than the commonly used methods. Our assay platform is suitable for TDM in standard care and in clinical studies for old and new ARVs.

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